REMARKS

In response to the Office Action mailed February 20, 2008, Applicants have amended claims 1 and 25. Claim 26 has been canceled and no new claims have been added. It is urged that support for all the above amendments may be found throughout the specification as originally filed, for example on page 17, lines 28-32 and page 21, lines 15-19. No new matter has been added. The above amendments are not to be construed as acquiescence with regard to the Examiner's rejections and are made without prejudice to prosecution of any subject matter removed or modified by this amendment in a related divisional, continuation or continuation-in-part application. Following the amendments, claims 1, 5, 7, 10-13, 15, 16, and 25 are pending in the application. Favorable reconsideration of the subject application is respectfully requested in view of the above amendments and the following remarks.

Amendments to the Claims

Applicant submits that claims 1 and 25 have been amended, without acquiescence, to recite "the signal moiety capable of providing a detectable signal when cleaved by substantially all non-target microorganisms in the sample." Support for this amendment can be found throughout the specification as filed.

Applicant has further amended claim 1 to recite "comprising a growth-supporting medium for the specific enrichment of a target microorganism." Support for this amendment can be found in the as-filed specification, for example, on page 17, lines 28-32, which recites that *Campylobacter* is used as a prototypical example and that one of ordinary skill in the art would readily conclude that the general scheme can be extended to all microorganisms. Additional support can be found on page 21, lines 15-19, which states that medium can be used for the specific enrichment of *Campylobacter*. One of ordinary skill in the art would interpret the above set of facts that apply to *Campylobacter* as an example, to encompass media that may specifically enrich the presence of any microorganism. Moreover, Applicant, in a previous response dated December 5, 2007, cited Novier *et al*., 2000. *Turk J Vet Anim Sci*, Vol. 24, p. 459-464 (*E.*

coli OH157), Brackett *et al.*, 1989. *International Journal of Food Microbiology,* Vol. 8, p. 219-223 and Kovacs *et al.*, 1991. *Acta Microbiol Hung.* 38(2), pp.141-5 (*Listeria*), Aldridge *et al.*, 1977. *Journal of Clinical Microbiology*, Vol.6, No. 4, p.406-413 (*Staphlococcus aureus*), and Vassiliadis *et al.*, 1981. *Applied and Environmental Microbiology*, Vol.42, No. 4, p. 615-618 (*Salmonella*) to support that the skilled artisan had knowledge of said media.

Furthermore, Applicant asserts that claim 1 has been amended to recite wherein said target microorganism is selected from the group consisting of *Salmonella*, *Listeria monocytogenes*, and *Campylobacter* . Applicant submits that *Listeria monocytogenes* is a well known species of the genus *Listeria*, and thus, one of ordinary skill in the microbiological arts would appreciate that Applicant was in possession of the *L. monocytogenes* species at the time of filing the instant application.

Applicant submits that the courts have held that all that is required to comply with the written description requirement is that the specification **reasonably convey** to persons skilled in the art that the inventor had possession of the subject matter claimed. (*In re Edwards*, 568 F.2d 1349, 1351, 196 USPQ 465, 467 (CCPA 1978) (emphasis added).) Applicant submits the specification is not required to describe the claim limitations exactly, "but only so clearly that persons of ordinary skill in the art will recognize from the disclosure that applicants invented [the subject matter], including those limitations." *In re Wertheim*, 541 F.2d 257, 262; 191 U.S.P.Q. 90, 96 (C.C.P.A. 1976). Furthermore, Applicant submits that the claims "need not be described in *haec verba* to satisfy the description requirement." *In re Smith*, 458 F.2d 1389, 59 C.C.P.A. 1025, 173 U.S.P.Q. 679 (1972).

Objections to the Claims

Claim 26 is objected to for allegedly being of improper dependent form, for failing to further limit the subject matter of a previous claim. Applicant, without acquiescence, has canceled claim 26; thus obviating this objection. Applicant respectfully requests that the Examiner withdraw this objection.

Claim Rejections Under 35 U.S.C. § 112,First Paragraph, Written Description

Claims 1, 5, 7, 13, 15, 16, and 25 stand rejected under 35 U.S.C. §112, first paragraph, as failing to comply with the written description requirement for allegedly claiming subject matter which was not described in the specification in such a way as to reasonably convey to the skilled artisan that the inventor, at the time the application was filed, had possession of the claimed invention. Specifically, the Examiner asserts that Applicant's disclosure allegedly fails to identify which aminopeptidases are specifically absent from the target microorganisms, but are present in all non-target microorganisms.

Applicant respectively traverses this basis of rejection and submits that the skilled artisan would readily understand that Applicant was in possession of the claimed invention at the time the application was filed.

Applicant submits that one having ordinary skill in the art was aware at the time of filing the instant application that several species of bacteria lack specific aminopeptidase activities (*i.e.*, the ability to hydrolyze particular aminopeptidase substrates). Applicant submits, for example, U.S. Patent No. 5,330,889 (Monget; see abstract and entire patent) as evidence that *Listeria monocytogenes* lacks glycine aminopeptidase activity, whereas all other members of the genus tested, possess this peptidase activity. In a similar example, *Listeria monocytogenes* lacks aminopeptidases activity for DL-alanine- and D-alanine- based substrates, whereas all other members of the genus tested, possess this peptidase activity (Clark and McLauchlin, *Journal of Clinical Microbiology*, Aug. 1997, pp. 2155-2156). Another example, Bennett *et al.*, *Letters in Applied Microbiology*,1999, Vol. 28, pp. 175-178 (see entire document, copy attached) discloses *Salmonella* does not possess pyrrolidonyl aminopeptidase activity, but that other bacteria capable of growing on the same enrichment medium (*e.g.*, *Citrobacter*) do possess this peptidase activity.

In view of the foregoing examples, the skilled artisan would readily conclude that Applicant was in possession of the entire claimed genus of aminopeptidases

that are absent from the target microorganism, wherein said target microorganism is selected from the group consisting of *Salmonella*, *Listeria monocytogenes*, and *Campylobacter*, wherein said substrate comprises a signal moiety, the signal moiety capable of providing a detectable signal when cleaved by substantially all non-target microorganisms in the sample, and wherein said conditionally detectable marker and said substrate for an aminopeptidase are not the same molecule.

Accordingly, Applicant respectfully requests the Examiner carefully reconsider and withdraw this basis of rejection.

Claim Rejections Under 35 U.S.C. § 112, First Paragraph, Enablement

Claims 1, 5, 7, 10-13, 15, and 16 stand rejected under 35 U.S.C. §112, first paragraph, as allegedly lacking enablement. Specifically, the Examiner asserts that while the specification is enabling for production and use of compositions for identifying a pure culture sample of Gram-negative bacteria such as *Campylobacter*, said compositions do not reasonably provide enablement for the production and use of compositions for detecting any target microorganism in any sample, or even for detecting *Campylobacter* in a mixed sample, or for differentiating between *Campylobacter* and any Gram-positive bacteria.

Applicant respectfully traverses these bases for rejection and submits that the as-filed specification provides ample disclosure and guidance to practice the entire claimed scope of the invention.

As an initial point, Applicant wishes to thank the Examiner for acknowledging the enablement of claim 25, but Applicant disagrees with the Examiner that the claim is only partially enabled. Applicant submits that the skilled artisan would recognize that claim 25 is fully enabled by the as-filed specification, especially in view of the following remarks.

The Examiner contends that the skilled artisan would need to engage in undue experimentation in order to practice the full breadth of the presently claimed invention. Applicant disagrees and submits that the present claims have been amended,

without acquiescence, to clarify that the signal moiety capable of providing a detectable signal when cleaved by **substantially** all non-target microorganisms in the sample. Thus, in order to determine the aminopeptidase that is substantially absent in the target bacteria, but present in the substantially all of the non-target bacteria, the skilled artisan would merely engage in routine experimentation, similar to that described in Peterson *et al.*, 1978, Kampfer, 1992, and Westley *et al.*, 1967 (already made of record), for example. Applicant submits that these are sample based systems, wherein the sample as a whole may be examined for aminopeptidase activities regardless of the bacterial flora present. Applicant has demonstrated aminopeptidases that are absent in the target bacteria were known in the art; thus, the skilled artisan would only need to determine the aminopeptidase activity present in substantially all the bacteria in the sample, (*i.e.*, not all known bacteria in existence). Applicant has demonstrated aminopeptidases that are absent in the target bacteria were known in the art; thus, the skilled artisan would only need to determine the aminopeptidase activity present in substantially all the bacteria in the sample, (*i.e.*, not all known bacteria in existence). Furthermore, Applicant submits that in most samples where contamination of biological samples is a concern (*e.g.*, poultry, beef), the skilled artisan is keenly aware of the type of bacterial pathogens that predominantly contaminate the sample (*e.g.*, poultry – *Campylobacter*; beef – *E. coli*). Moreover, this rationale is demonstrated in Example I of the as-filed application, wherein Applicant tested poultry wash samples for *Campylobacter* contamination and not for contamination by irrelevant and/or non-pathogenic bacteria. However, Applicant has amended the claims to recite wherein the target microorganism is *Salmonella*, *Listeria monocytogenes*, or *Campylobacter*. Furthermore, Applicant has provided examples of the skilled artisan's knowledge that the aminopeptidase substrates that are substantially absent in each of these bacteria, are present in substantially all non-target bacteria in the sample that are capable of growing on the specific enrichment medium appropriate for the given target bacterium.

Applicant notes that the skilled artisan's knowledge of growth supporting media for the specific enrichment of the target bacterium would result in the majority of

microorganism growing on such media to be target microorganisms. Applicant submits that Figure 1 provides evidence that the number of false positives in the poultry wash samples are negligible (*i.e.*, most colonies are target microorganisms, **and not** Gram-positive bacteria or other non-target bacteria) by demonstrating that the number of *Campylobacter* in said samples is the same, whether determined by the Applicant's method or the method approved by the U.S. government, wherein the presence of *Campylobacter* is confirmed by agglutination assays. Applicant has also produced a commercial embodiment for the specific detection of *Campylobacter* in mixed bacterial samples (http://www.biocontrolsys.com/products/simcamp.html). The foregoing examples also provide support for the contention that the claims specific enrichment media preferably allows the target microorganism to grow and "out-compete" the non-target microorganisms **in the sample**.

Furthermore, Applicant submits Exhibits A-C to support the art accepted definition of specific enrichment of a target bacterium. Exhibit A is an excerpt from the glossary of a new online microbiology textbook available at the Univ.of Wisconsin-Madison Bacteriology Dept. (http://www.microbiologytext.com/index.php?module=Book&func=displayglossary), which states:

> "enrichment medium- medium formulated to encourage the growth of a desired microbe, while inhibiting the growth of other microbes."

Exhibit B is an excerpt from a lab manual published at the Univ. of California at Fresno available online at http://blc.biolab.udel.edu/Harding/308.pdf, which states:

> "Enrichment culture means that you incubate liquid medium under conditions that favor the growth of the organism of interest. Other bacteria may also grow, but if your conditions are sufficiently restrictive, the desired organism will predominate."

Exhibit C is an excerpt from a chapter of an online textbook published by the Department of School Education, Government of Tamil Nadu, India available at http://www.textbooksonline.tn.nic.in/Books/11/MicroBio-EM/chapter%205.pdf, which states:

> "Enrichment medium - Enrichment medium is a liquid medium which enhances the growth of certain bacterial species, while inhibiting the growth or prolonging the lag phase of unwanted organisms thus altering the ratio between the two in favor of the required bacterial species."

Applicant submits that one having ordinary skill in the art, in view of the art accepted understanding of the specific enrichment of bacteria; the evidence supplied in Figure 1; and a commercial embodiment of the presently claimed invention; would readily conclude that a growth supporting media for the specific enrichment allows the preferentially growth of the target bacterium and not non-target bacteria.

The Examiner contends that when the presently claimed assay is used to identify *Campylobacter*, Gram-positive bacteria will give rise to the same signals as presumptive and confirmation indicators as *Campylobacter*. Applicant disagrees with the Examiner and points to the discussion above, wherein the skilled artisan would use a growth supporting media for the specific enrichment of *Campylobacter*, which would exclude the growth of Gram-positive organisms, as such media are commonly known to one of ordinary skill in the microbiological arts.

Accordingly, Applicant submits that in view of the foregoing remarks and amendments, the skilled artisan would readily be able to practice the full scope of the presently claimed invention without undue experimentation. Reconsideration and withdrawal of this basis for rejection is respectfully requested.

Claim Rejection under 35 U.S.C. § 103(a)

Claims 1, 5, 7, 10-13, 15, 16, and 25-26 stand rejected under 35 U.S.C. § 103(a) as allegedly unpatentable over Manafi *et al.* (J. Applied Bacteriology, 1990) in view of Molina *et al.* (Enfermedades Infecciosas y Microbiologia Clinica, 1991) and Tuompo *et al.* (U.S. Patent No. 5,420,017). Specifically, the Examiner contends that Manafi *et al.* disclose a method and composition for detecting Gram-negative bacteria in a sample using AAMC. Further, the Examiner contends that Tuompo *et al.* disclose a method and kit for detecting microorganisms in a sample using tetrazolium red. The Examiner alleges that the skilled artisan would find it obvious to combine the teachings of Manafi *et al.* with Tuompo *et al.* to arrive at the presently claimed composition.

Applicant traverses these bases for rejection and submits that one of ordinary skill in the art would not find it obvious to combine the references cited by the Examiner to arrive at the presently claimed invention. Thus, the Examiner has failed to establish a *prima facie* case of obviousness against the presently claimed invention.

The Examiner alleges that the skilled artisan would find it *prima facie* obvious to modify the composition of Manafi *et al.*, which includes AAMC in an agar plate, to further include tetrazolium red, as taught by Tuompo *et al.* The Examiner contends that including a viability marker in the agar plate of Manafi *et al.* would provide an additional confirmation means of bacterial viability. Applicant respectfully submits that one of ordinary skill in the art would not include a viability dye for bacteria cultured on the **non-selective** media of Manafi *et al.*. If no colonies grow when cultured in the presence of non-selective media, how will using tetrazolium red tell one skilled in the art whether the bacteria were dead or the plate was faulty? If a non-selective plate is full of bacterial colonies, why would one of skill in the art dedicate time and money to add tetrazolium red to the growth medium, when he expects all bacteria to grow? In this scenario, the skilled artisan is spending resources to verify the growth of true negative colonies. Applicant submits that the skilled artisan would not reasonably dedicate such resources to verify truly negative results, it would simply be viewed as wasteful. Thus, Applicant submits that the inclusion of tetrazolium red in the embodiment contrived by the Examiner would not be obvious, let alone in the presently claimed invention, wherein the dye serves an important part in marking true positive colonies.

Applicant respectfully submits that neither Manafi *et al.*, Molina *et al.*, or Tuompo *et al.* alone or in combination, can detect a target microorganism from a mixed sample of microorganisms based on a positive signal from a viability marker and the absence of signal from an aminopeptidase substrate, wherein the target bacterium lacks sufficient aminopeptidase activity specific to hydrolyze said substrate. Applicant submits that the absence of bacterial culture in a growth supporting medium for the specific enrichment of the target bacterium renders the Examiner's embodiment inoperable for the purpose of detecting said target microorganism.

As explained above, in the reply to the written description and enablement rejections, Applicant has provided numerous lines of evidence to support that the specific

enrichment of a target bacterium results in the primary enrichment of the target bacterium and not the non-target bacteria present **in the sample**. Thus, when grown in the presently claimed media, the majority of bacteria present are target bacteria, which are marked by the viability dye, tetrazolium red. From these bacteria, only a minority of the growing bacteria are expected to be non-target bacteria, and thus, capable of hydrolyzing the aminopeptidase substrate of the presently claimed invention. The difference between the number of colonies marked with the viability dye and the number of colonies showing fluorescence as a result of cleaving the aminopeptidase substrate allows a false positive calculation, and moreover, the reliable estimation of target bacteria in the sample being tested. Applicant has provided proof-of-principle experiments summarized in Figure 1 as well as a commercial embodiment that relies on precisely these principles. These examples demonstrate Applicant includes a medium for specific enrichment; thus restricting growth of all non-*Campylobacter* organisms, Gram-negative or Gram-positive.

In contrast, in the embodiment contrived by the Examiner will not be operable for the detection of the presently claimed target bacteria, wherein the sample in said embodiment is not cultured on or in a growth medium for the specific enrichment of a target bacterium. One of ordinary skill in the art would recognize that bacterial growth on a non-selective or non-enriching medium does not impair the growth of one species (*e.g.*, non-target bacteria) with respect to another (*e.g.*, target bacterium). Thus, assuming *arguendo*, that a viability dye was included in the composition of Manafi *et al.*, said composition would still not function to detect the presently claimed target bacterium. For example, on a non-enrichment medium most bacterial colonies would be from species other than the target bacterium, but all colonies would be red. The number of colonies showing fluorescence from cleavage of the aminopeptidase substrate would far outweigh the number of target bacteria, or in the case of *Campylobacter*, target bacteria and Gram-positive bacteria. This is strictly due to the fact that no medium for specific enrichment of the target bacteria was included in the composition contrived by the Examiner. The skilled artisan would immediately appreciate that such an embodiment does not lead one skilled in the art to estimate the number of target bacteria in the sample; thus, the Examiner's embodiment is not operable for the intended use of the presently claimed compositions.

Applicant submits that one having ordinary skill in the art would not find it obvious to combine a redundant viability dye on a non-selective growth medium in order to identify a target bacterium based on the absence of an aminopeptidase activity as presently claimed. As explained above, the skilled artisan would immediately comprehend such a composition does not and can not reliably identify a target bacterium from a sample comprising numerous bacterial species. Thus, one having ordinary skill in the art would not reasonably expect to successfully identify a target bacterium by combining the references of Manafi *et al*., Molina *et al*., and Tuompo *et al*. and could not arrive at the presently claimed invention in view of these references.

Accordingly, Applicant submits that the Examiner has failed to establish a *prima facie* case of obviousness against the presently claimed invention. Reconsideration and withdrawal of this basis of rejection is respectfully requested.

The Director is authorized to charge any additional fees due by way of this Amendment, or credit any overpayment, to our Deposit Account No. 19-1090.

All of the claims remaining in the application are now believed to be clearly allowable. Favorable consideration and a Notice of Allowance are earnestly solicited.

Respectfully submitted,

SEED Intellectual Property Law Group PLLC

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WTC:jto

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Exhibits A-C

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membrane proteins and is also involved in protein secretion.

endosome

The term that describes a membrane-lined particle in a cell that has been taken up by endocytosis

endospores

A resting strucure produced by Bacillus, Clostridium and other species that is extremely resistant to heat, chemicals, radiation and drying

endotoxin

Lipopolysaccharide. Part of the gram-negative cell wall that when released from a pathogen has systemic toxic effects

enrichment culture

Culturing methods that favor the growth of one class of microbes over all others. Nutrients or growth parameters are manipulated to either encourage the growth of the desired class or discourage the undesirable microbes.

enrichment media

Media that selects for the growth of a certain desired class of microorganisms. Nutrients and incubation conditions are manipulated in such a way that the growth of a desired class of microbe in encouraged, or the growth of undesired microbes is inhibited.

enrichment medium

Medium formulated to encourage the growth of a desired microbe, while inhibiting the growth of other microbes

enthalpy

The sum of the internal energy of a system. The heat of a system.

entropy

A measure of the disorder or randomness of a closed system.

envelope

A term used to described the outer membrane of a virus

environmental microbiology

Environmental microbiology is the study of the numbers and species present in the various environments of the earth and their physiology.

Exhibit B

[1]308. ENRICHMENT AND ISOLATION OF *THIOBACILLUS*

E. Harding and E. Holbert

Lithotrophs ("rock-eaters") are bacteria that use reduced inorganic compounds as their energy source. Energy sources used by various lithotrophs include H_2, NH_4^+, NO_2^-, H_2S and S. Metabolism in lithotrophs is similar to that in humans and other **organotrophs** (organisms that use organic molecules as the energy source) in that both types of **chemotrophs** obtain energy by oxidizing chemicals. Chemotrophs can be contrasted with the light-using **phototrophs** that are studied in most of the activities in this unit.

Like most phototrophs, lithotrophs can use CO_2 as their carbon source. Plants and lithotrophs are **autotrophs**, organisms that can fix CO_2, while animals and other organotrophs are **heterotrophs**, requiring preformed organic carbon for biosynthesis.

Table 1. Terminology to describe carbon and energy sources used by organisms.

Metabolic class	Energy Source	Carbon Source	Example
chemotrophs	chemicals		
organotrophs	organic chemicals	organic chemicals	humans
lithotrophs	inorganic chemicals	CO_2	*Thiobacillus*
phototrophs	light		
photoautotrophs	light	CO_2	plants
photoheterotrophs	light	organic chemicals	some bacteria

In this exercise, you will attempt to enrich for and isolate lithotrophic bacteria that use reduced sulfur compounds as the energy source. These bacteria, called *Thiobacillus*, obtain energy by "burning" compounds such as H_2S, S and $S_2O_3^{2-}$ with oxygen, producing more oxidized forms of sulfur, including H_2SO_4 (sulfuric acid), as waste products. *Thiobacillus* is found in soil, water, and pond sediments; anywhere O_2 and reduced forms of sulfur meet.

Thiobacillus species are ecologically and economically significant. When coal or mineral ores with a high sulfur content are excavated and exposed to atmospheric oxygen, these bacteria can grow, producing sulfuric acid and resulting in acidic mine drainage waters. Some miners actually exploit *Thiobacillus*, piling up and watering low-grade ores and allowing the sulfuric acid produced by the bacteria to dissolve the minerals from the ore. Minerals such as copper are then reclaimed from the leachate which is recycled back to the top of the ore pile. *Thiobacillus* also has some use in agriculture. Alkaline soils, such as those on the west side of the San Joaquin Valley, can be neutralized by adding elemental sulfur and allowing the bacteria to oxidize it to sulfuric acid.

Enrichment culture means that you incubate liquid medium under conditions that favor the growth of the organism of interest. Other bacteria may also grow, but if your conditions are sufficiently restrictive, the desired organism will predominate. You can then subculture a small

[1]Allow two weeks for incubations and subculturing.

amount of your first enrichment into a fresh flask of medium, in hopes that the organism you are looking for will become even more predominant. Several serial subcultures may be followed by an attempt to streak the bacterium for isolation on solid medium (See "Aseptic Technique" in Appendix A).

The medium for enrichment of *Thiobacillus* contains $Na_2S_2O_3$ as the energy source, along with various inorganic nutrients required for synthesis of cell material. The atmosphere over the flask provides CO_2 as a carbon source and O_2 with which to oxidize the $Na_2S_2O_3$. The flask is incubated in the dark to prevent growth of phototrophs. There is no organic carbon in the medium, so the only food source for heterotrophs is organic material released from live or dead *Thiobacillus* cells.

Text References

POH 392, Comparative Metabolism; 466, Nutritional Categories of Bacteria

Study Questions

Define the terms: C-autotroph, heterotroph, phototroph, organotroph, lithotroph.
How does *Thiobacillus* make its living? What is the ecological significance of this bacterium?
Explain how enrichment culture works.

Purpose

To enrich for and isolate *Thiobacillus* and to demonstrate lithotrophic growth.

Materials: 2 flasks *Thiobacillus* enrichment medium, 50 ml/250 ml flask; 2 plates *Thiobacillus* agar; 1 tube *Thiobacillus* enrichment medium, 5 ml/18 mm tube

Obtain soil, water, or pond sediment from an area likely to contain *Thiobacillus* species. Soil from a vineyard which has been treated with sulfur is a good potential source. Ponds with black sediments are likely to have *Thiobacillus* in the water or at the sediment surface. (The sediments are black because they contain H_2S.) Different students should use different source material. Enrichment culture of bacteria is a gamble, and the more different sources are used, the higher the probability that someone will isolate the bacteria.

Add 2-3 g of soil or 5 ml of water to one flask of medium. Incubate at 30° C for a few days. Check every two days for a decrease in pH or the formation of a layer of bacteria and free sulfur at the surface of the medium. You can pour the culture into a clean beaker, check the pH with a pH meter and pour the culture back into the flask. If the pH is between 5 and 6, you should subculture to a flask of fresh medium. If you wait too long, the bacteria may produce enough sulfuric acid to kill themselves.

To subculture, inoculate one drop of the enrichment into a flask of fresh medium. Substantial growth should occur in the second enrichment in 24 h. *Record on the worksheet how long you incubated the cultures and what the pH values were when you checked them.*

Streak for isolation ("Aseptic Techniques" in Appendix A) on a plate of *Thiobacillus* agar. Incubate the plates at 30° C for a few days. Restreak as necessary to obtain well-isolated colonies.

Inoculate a well-separated colony into the tube of *Thiobacillus* medium. If you have a pure culture of *Thiobacillus*, growth should occur within a few days.

Gram stain a smear of your bacteria as described in Box 1 and examine the stained smear under the microscope. The Gram stain (POH Box 21.A) differentiates bacteria into two groups (gram-positive and gram-negative) based on cell wall structure. *Record your results on the worksheet.*

Box 1. The Gram stain procedure.

To make a smear, spread a loopful of bacteria onto a microscope slide. Allow the smear to air-dry completely. Then pass the slide briefly through a Bunsen burner flame, smear side up, two or three times to heat fix the smear. Heat fixing makes the bacteria adhere to the slide. Do not overheat the slide; you should be able to touch it to the back of your hand without discomfort.
To Gram stain, place your slide on a staining rack over the sink. Put a few drops of crystal violet over the smear. Allow the stain to sit for 1 minute, then rinse with gently running water. Put a few drops of iodine over the smear. Again allow it to sit for 1 minute, then rinse with water. Now tilt the slide at a 45° angle. Pour 95% ethanol over the smear until the drops running off the end of the slide are no longer colored. This should take 10 to 20 seconds. Rinse with water. Replace the slide on the rack. Cover the smear with a few drops of safranin and allow it to sit for 1 minute. Gently rinse the excess safranin from the slide with water.
Ask you instructor to assist you to examine the smear at 1000X using an oil-immersion lens.
Crystal violet and iodine form a complex in the cells. Gram-positive bacteria retain the crystal violet-iodine complex during the ethanol rinse and remain blue-purple. They retain the dye complex because their cell walls contain a thick, tight mesh of a structural molecule called peptidoglycan. Gram-negative bacteria have a thin, loose peptidoglycan mesh, so they lose the crystal violet-iodine complex and are colorless after the alcohol treatment. Counter-staining with safranin makes gram-negative bacteria visibly pink, while not affecting the purple color of the gram-positive bacteria.

Cleanup

Use alcohol to remove the labels from all glassware. Discard tubes and flasks of medium in the microbiology discad area. Tubes should be arefully placed in a slanted basket to avoid spilling. Discard plastic Petri dishes in the BIOHAZARD bag.

Recipes

Thiobacillus enrichment broth

NH_4Cl	1.0 g/l	N source
$MgCl_2 \cdot 6H_2O$	0.5 g/l	Mg source
$MgSO_4$	0.3 g/l	Mg source
KH_2PO_4	0.4 g/l	buffer and P source
K_2HPO_4	0.6 g/l	buffer and P source
$CaCl_2 \cdot 2H_2O$	0.2 g/l	Ca course
$FeCl_3 \cdot 6H_2O$	0.02 g/l	Fe source
$Na_2S_2O_3 \cdot 5H_2O$	10 g/l	energy source
Trace element solution	10 ml/l	various cofactors for enzymes

Trace element solution

$CaCl_2 \cdot 2H_2O$	100 mg/l
$ZnSO_4 \cdot 7H_2O$	88 mg/l
$CuSO_4 \cdot 5H_2O$	40 mg/l
$MnSO_4$	15 mg/l
$Na_2B_4O_7$	10 mg/l
$(NH_4)_6Mo_7O_{24} \cdot 4H_2O$	5 mg/l

Sterilize separately the phosphates, thiosulfate and trace elements.

Additional References

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Kelly, D. P. and A. P. Harrison. 1989. *Thiobacillus*, p. 1842-1858 *In* J. T. Staley, M. P. Bryant, N. Pfennig and J. G. Holt (Ed) Bergey's Manual of Systematic Bacteriology. Williams and Wilkins, Baltimore.

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308. ISOLATION OF *THIOBACILLUS* (13 PTS)

Name . Lab day and time _____

PRELAB PREPARATION:

1. Procedural outline:

2. Where will you get your sample? What suggests to you that *Thiobacillus* is present in this sample?

3. What is the purpose of subculturing into a second flask of *Thiobacillus* medium? Of streaking onto a plate of *Thiobacillus* agar? (See "Aseptic Technique" in Appendix A.)

RESULTS:

4. First Flask: Date inoculated_____

date					
pH					

observations:

5. Second Flask: Date inoculated_____

date				
pH				
growth				

observations:

6. Streak Plate:

7. Broth Tube:

8. Gram stain:

9. Was *Thiobacillus* present in your sample? What experimental evidence supports your answer?

10. Compare your success (or lack thereof) with the results obtained by other students who used different sources.

11. Suggest possible sources of error in this experiment.

12. Describe another lithotroph of ecological importance.

Exhibit C

Chapter 5

PURE CULTURE METHODS

In the natural environments microorganisms exist in mixed cultures. To establish the role of microbial agent to a disease process, it is essential to demonstrate the organisms or its components in the diseased tissues. To accomplish this, the organism must be cultivated from the tissues. Similarly to know the kinds of organism present in the environment it is necessary to grow them in artificial media. Cultivation of the organism is also essential to obtain pure culture of clone of cells derived from a single cell to perform biochemical differentiation tests and **susceptibility tests since mixed cultures give misleading results.**

Artificial culture media

A medium is an environment which supplies the ingredients necessary for the growth of the organism. Various kinds of media have been prepared in the laboratory to isolate, grow and identify an organism. Depending on the need to isolate and identify an organism from a particular sample or environment, different kinds of media are formulated.

Kinds of media

Basal or supportive media

Basal medium is one that contains nutrients that allow the growth of most nonfastidious organism without affording growth advantage to any particular organism over others. Example is *Nutrient agar,* and *Trypticase Soy agar.*

Enrichment medium

Enrichment medium is a liquid medium which enhances the growth of certain bacterial species, while inhibiting the growth or prolonging the lag phase of unwanted organisms thus altering the ratio between the two in favor of the required bacterial species. Example is Selenite F broth for the isolation of Salmonella from stool.

To get a pure culture of the organism, any one of the solid media mentioned above is used. In order to get discrete separate colonies, the surface of the medium must be dry. The material is inoculated on the surface by spreading with a sterile loop in such a way that bacteria are ultimately deposited singly. When the bacteria are at a sufficient distance from each other, the whole progeny of each accumulates locally during growth to form a discrete mass or colony which is readily visible to the naked eye. Each colony is presumed to be a pure culture, consisting exclusively of the descendants of a single cell. It may be picked up with a sterile wire to prepare a pure subculture in a fresh medium.

Growth and colony characteristics of Bacteria

The appearances of growths of bacteria in liquid media are generally not distinctive. There is a uniform turbidity in the liquid and little deposit at the bottom. Colony morphology of the isolated bacteria on the solid media has much more value. Attention is paid to the *size* of the colony (diameter in mm), their *outline*, whether circular and entire or indented, or wavy or rhizoid, their elevation low convex, high convex or flat plateau-like, umbonate or nodular, their *translucency*, whether transparent, translucent, or opaque, their *pigmentation,* colorless, white or otherwise pigmented, and whether they produce any *change in the medium* (haemolysis in a blood-containing medium).

Example: Colony characteristics of *Staphylococcus aureus* on Nutrient agar

After aerobic incubation at 37°C for 24 hours, colonies are 1-3 mm in diameter and have a smooth glistening surface, an entire edge, a soft butyrous consistency and an opaque, pigmented appearance.

Growth characteristics of yeasts

Yeasts are grown on Sabouraud Dextrose agar aerobically. Yeasts grow as typical pasty colonies and give out yeasty odor. The colony morphology varies with different yeasts.

Growth characteristics of filamentous fungi

The most common medium used for the isolation of fungi is Sabouraud Dextrose agar. While observing colony morphology, one must note the colors of the surface and the reverse of the colony, the texture of the surface (powdery, granular, woolly, cottony, velvety or glabrous), the topography (elevation, folding, margins, etc) and the rate of growth.

EXERCISE

Points to remember

1. Existence of organism in nature as mixtures
2. Need to isolate them in pure culture
3. Growth of microbes in different media

Self evaluation

1.Why should the organism be grown in pure culture?
2. Define a culture medium
3. Classify different kinds of culture media and give one example
4. Define basal medium and give one example
5. Define enriched medium and give two examples
6. Define differential medium and give two examples
7. Define selective medium and give one example
8. Define enrichment medium and give one example
9. Describe the growth characteristics of bacteria
10. Describe the growth characteristics of yeast and filamentous fungi.